

The activity state of factor VII in plasma

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The activity state of factor VII in plasma: two pathways for the cold promoted activation of factor VII

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SUMMARY. The apparent amount of factor VII as determined in a one-stage test depends on the type of thromboplastin used: bovine thromboplastin only reacts with human factor VII_a whereas human thromboplastin interacts with unactivated human factor VII as well. Therefore the ratio factor VII activity as measured with bovine thromboplastin divided by the factor VII activity as assessed with human thromboplastin reflects the state of activation of factor VII in plasma. This approach was used to study the process of cold promoted factor VII activation and the involvement of different clotting factors therein. It could be shown that cold promoted activation does not occur in the absence of factors II and XII and is reduced for about 50% in factor IX deficient plasma. The other coagulation factors have a minor influence on the process. The results indicate that the cold promoted factor VII activation is the result of activation by both activated contact products and thrombin.

Factor VII activation has been described to occur in circulation plasma in different pathological situations (Meade *et al*, 1980; Meade, 1983; van Deijk *et al*, 1983a, b). We therefore used the test described by Hemker *et al* (1976) to measure state of activation of factor VII in plasma.

Human factor VII is a single chain protein that can be converted to factor VII_a, a two chain protein. This conversion is associated with an increase in activity as measured in a one-stage test. The increase, however, is dependent on the type of thromboplastin used to initiate the reaction. Bovine thromboplastin interacts with human factor VII_a but hardly with human factor VII while human thromboplastin interacts with both human factor VII and factor VII_a. The increase in activity with conversion of the single chain into the double chain form will therefore be much more pronounced when tested with bovine thromboplastin than with

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human thromboplastin. Therefore the state of activation of factor VII in plasma can be expressed by the ratio: factor VII_{bov}/factor VII_{hum} (bov = estimated with bovine thromboplastin; hum = estimated with human thromboplastin). This test was used as a tool to investigate the pathway(s) that lead(s) to cold promoted factor VII activation.

MATERIALS AND METHODS

All chemicals used are of reagent grade; all solutions are made in distilled water; Michaelis buffer: 0.15 M Veronal/acetate, pH 7.4. Hirudin is obtained from Sigma. Thrombin is obtained from Serva. Chromogenic substrate S2302 is obtained from Kabi Vitrum.

Factor VII-deficient reagent is prepared as described by Hemker *et al* (1972). The artificial reagent contains: 64% human factor X, 72% bovine factor V, 74% human factor II and 2.25 g/l bovine fibrinogen. The factor VII concentration in the reagent is 1.68%.

Thromboplastins. Human and bovine thromboplastins are prepared as described by van Dam-Mieras *et al* (1984) and stored in small portions at -20°C. Prior to use thromboplastin solutions are prewarmed at 37°C for minimally 20 min.

Collection and treatment of samples. Blood is collected by venipuncture without stasis, in plastic tubes containing Na-citrate (9 volumes of blood + 1 volume of 0.109 M Na-citrate). The first few millilitres of blood are discarded to avoid activation by tissue factor.

Platelet poor plasma is obtained from fresh blood by centrifugation (15 min at 13 000 g).

Platelet free plasma is obtained by centrifugation of platelet poor plasma (20 min at 20 000 g at 4°C) and stored in 1 ml portions at -80°C until use.

Pooled normal plasma is obtained by pooling equal amounts of platelet free plasma from at least 30 healthy individuals (15 males and 15 females, not using oral contraceptive agents, average age 30 years) and stored in small portions at -80°C until use.

The following samples are used: pooled human normal plasma; congenital deficient plasmas: V deficient ($n=2$), VII deficient ($n=2$), VIII deficient ($n=6$), IX deficient ($n=3$), X deficient ($n=3$), XI deficient ($n=2$), XII deficient ($n=2$). In the results mean values of the data obtained with the individual factor deficient plasmas are given.

Incubation of plasma at 4°C. Immediately after thawing, plasma samples are incubated at 4°C in glass or plastic tubes. After 0, 1, 2, 4, 24 and 48 h subsamples are taken from the incubates and, after dilution with Michaelis buffer, screened for factor VII activation.

During the incubation at 4°C the concentration of fibrinogen in and the pH of the incubation mixtures were constant.

Determination of factor VII activation. Pipette successively into a glass tube: plasma sample diluted with Michaelis buffer, 0.1 ml; factor VII deficient reagent, 0.1 ml; human or bovine thromboplastin, 0.1 ml; incubate 30 s at 37°C; CaCl₂ (33 mM), 0.1 ml; register the clotting time.

The clotting time obtained with the sample is compared to a reference curve or a reference table derived from the reference curve using a computer program (Hemker *et al*, 1972). The reference curve is obtained by measuring the clotting times of a series of dilutions of pooled normal plasma; the clotting times obtained with these standard plasma dilutions are plotted against the factor VII concentrations, expressed in per cent of pooled normal plasma.

according to Hemker *et al* (1972). Reference curves for both the human and the bovine test system are constructed.

Prekallikrein activation is measured as described by Alving *et al* (1983) using the chromogenic substrate S2302.

RESULTS

Fig 1 shows the results obtained when pooled normal plasma is incubated at 4°C in glass and plastic tubes. The apparent amount of factor VII as measured with a test system using bovine thromboplastin (we shall call this test system the bovine test system) shows a five-fold increase during incubation at 4°C. The apparent amount of factor VII as determined in a one-stage test using human thromboplastin (the human test system) also increases during incubation at 4°C but to a lesser degree (about two-fold). It can also be concluded that, although the time course of factor VII activation during incubation at 4°C in glass and plastic tubes is different, the ultimate stage of factor VII activation is very similar in both materials. This also follows from Fig 2 in which the ratio factor VII_{bov}/factor VII_{hum} is plotted.

The involvement of other clotting factors in the process of cold promoted factor VII activation has been studied by incubating congenital factor deficient plasmas at 4°C, again in glass and plastic tubes. The results are shown in Figs 3–6. It can be concluded that cold promoted activation of factor VII only takes place when the factors XII and of course VII are present in the plasma. In the absence of factor IX cold promoted activation of factor VII is reduced to about 50%. In factor VIII deficient plasma the results obtained in glass are similar

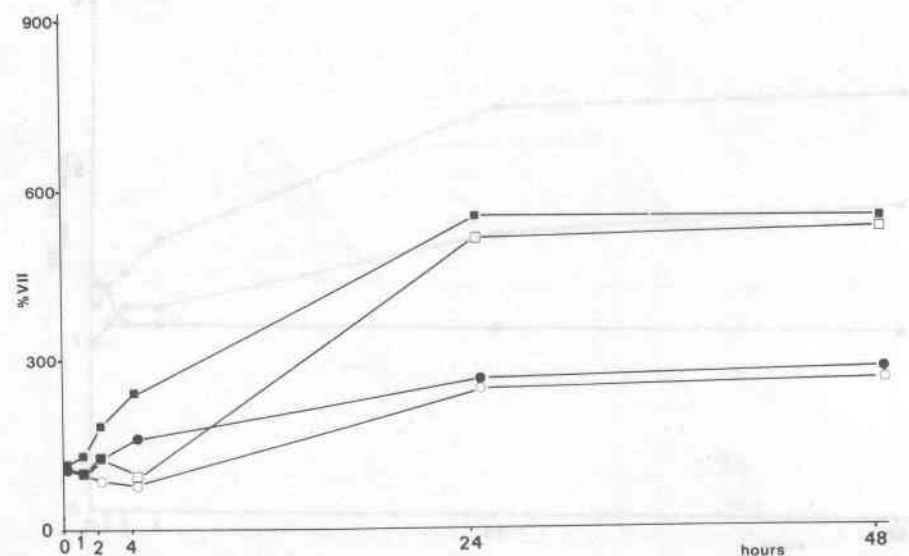


Fig 1. Activation of factor VII in normal plasma during incubation at 4°C in glass and plastic tubes as measured with bovine and human thromboplastin. ■, Glass, bovine thromboplastin; □, plastic, bovine thromboplastin; ●, glass, human thromboplastin; ○, plastic, human thromboplastin.

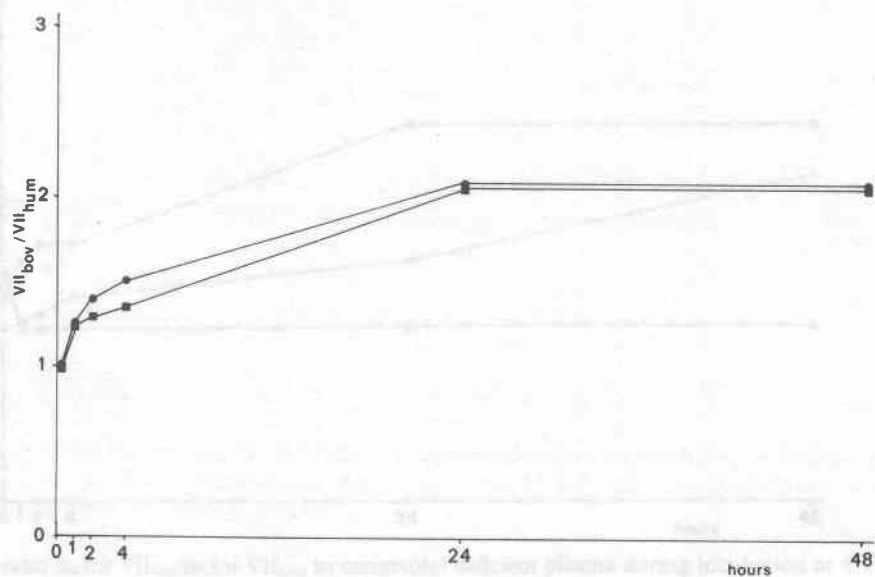


Fig 2. The ratio factor VII_{bov}/factor VII_{hum} in pooled normal plasma during incubation at 4°C in glass (●) and plastic (■) tubes.

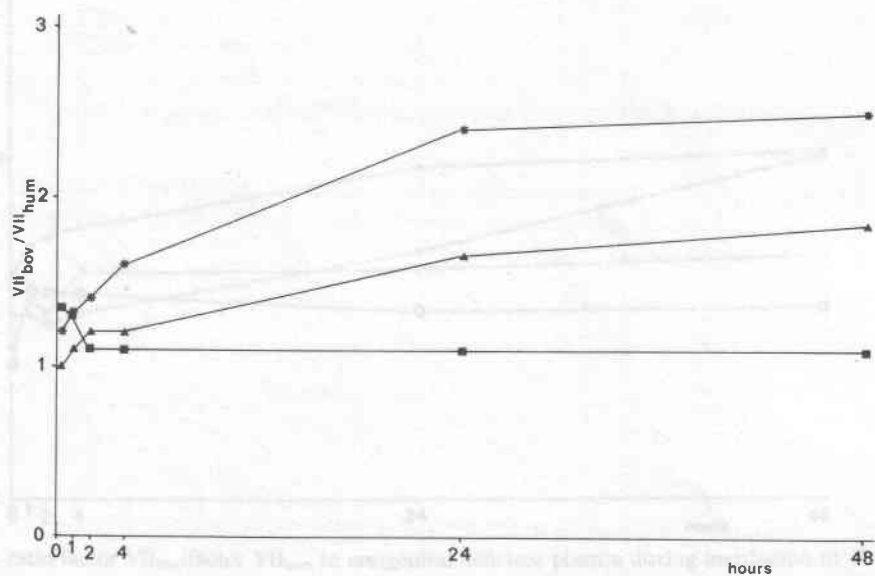


Fig 3. The ratio factor VII_{bov}/factor VII_{hum} in congenital deficient plasma during incubation at 4°C in glass tubes. ▲, Factor V deficient plasma; ■, factor VII deficient plasma; *, factor X deficient plasma.

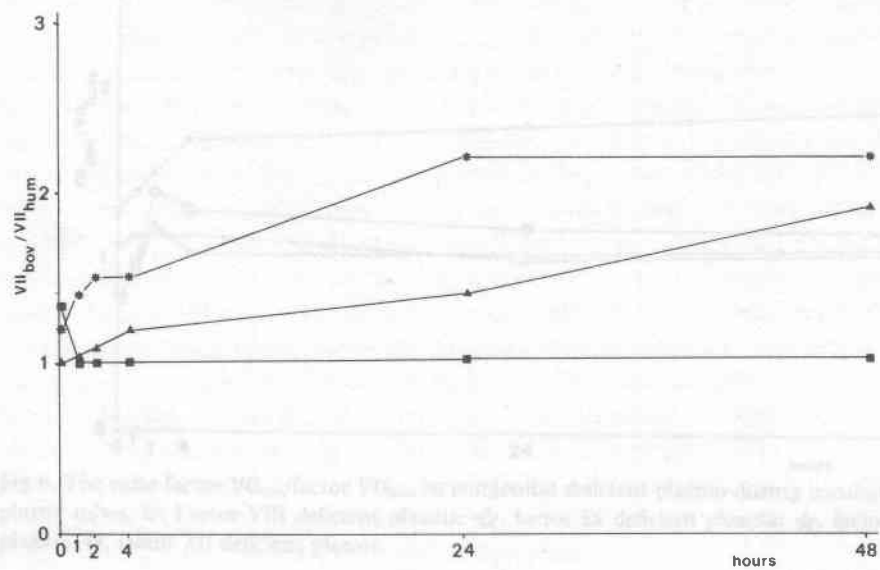


Fig 4. The ratio factor VII_{bov}/factor VII_{hum} in congenital deficient plasma during incubation at 4°C in plastic tubes. ▲, Factor V deficient plasma; ■, factor VII deficient plasma; *, factor X deficient plasma.

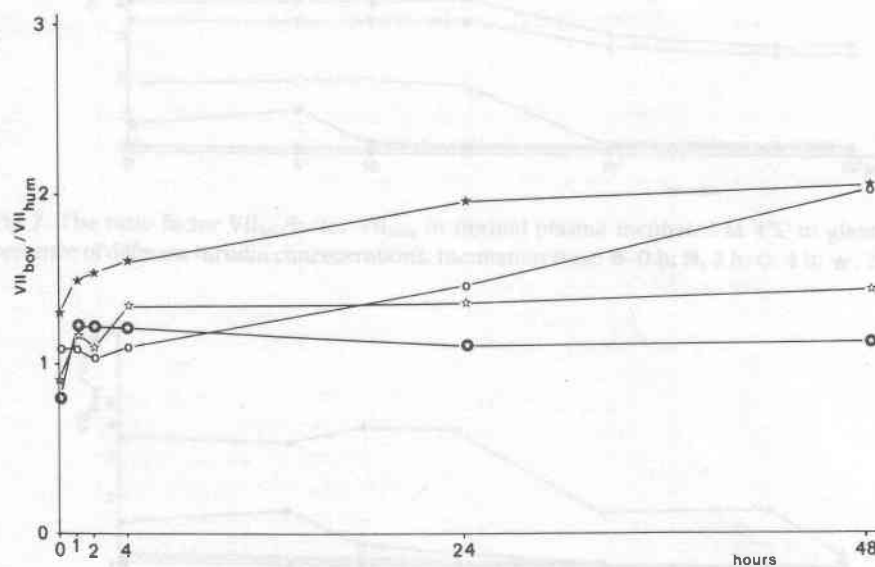


Fig 5. The ratio factor VII_{bov}/factor VII_{hum} in congenital deficient plasma during incubation at 4°C in glass tubes. ○, Factor VIII deficient plasma; ☆, factor IX deficient plasma; ★, factor XI deficient plasma; ●, factor XII deficient plasma.

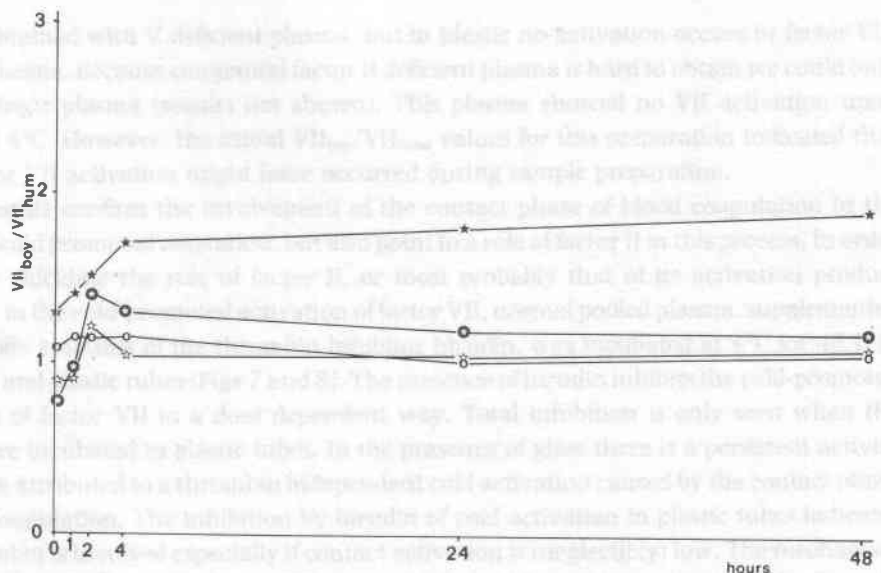


Fig 6. The ratio factor $VII_{bov}/factor VII_{hum}$ in congenitally deficient plasma during incubation at 4°C in plastic tubes. ○, Factor VIII deficient plasma; ☆, factor IX deficient plasma; ★, factor XI deficient plasma; ●, factor XII deficient plasma.

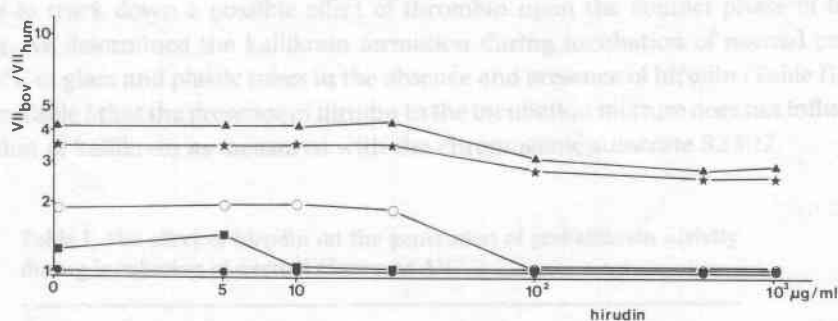


Fig 7. The ratio factor $VII_{bov}/factor VII_{hum}$ in normal plasma incubated at 4°C in glass tubes in the presence of different hirudin concentrations. Incubation time: ●, 0 h; ■, 2 h; ○, 4 h; ★, 24 h; ▲, 48 h.

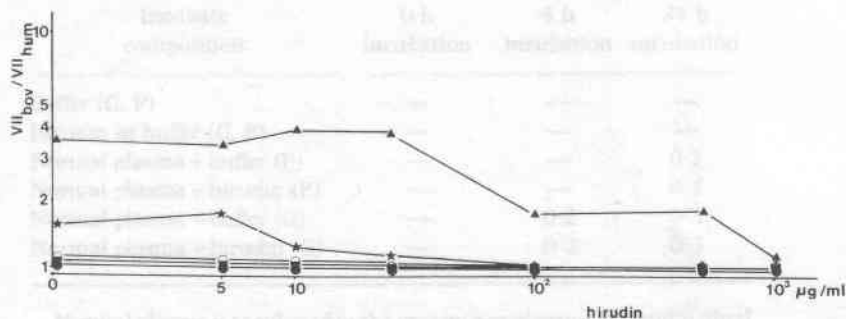


Fig 8. The ratio factor $VII_{bov}/factor VII_{hum}$ in normal plasma incubated at 4°C in plastic tubes in the presence of different hirudin concentrations. Incubation time: ●, 0 h; ■, 2 h; ○, 4 h; ★, 24 h; ▲, 48 h.

to those obtained with V deficient plasma, but in plastic no activation occurs in factor VIII deficient plasma. Because congenital factor II deficient plasma is hard to obtain we could only test one single plasma (results not shown). This plasma showed no VII activation upon storage at 4°C. However, the initial $VII_{\text{bov}}/VII_{\text{hum}}$ values for this preparation indicated that some factor VII activation might have occurred during sample preparation.

The results confirm the involvement of the contact phase of blood coagulation in the process of cold promoted activation, but also point to a role of factor II in this process. In order to further elucidate the role of factor II, or most probably that of its activation product thrombin, in the cold-promoted activation of factor VII, normal pooled plasma, supplemented with various amounts of the thrombin inhibitor hirudin, was incubated at 4°C for 48 h in both glass and plastic tubes (Figs 7 and 8). The presence of hirudin inhibits the cold-promoted activation of factor VII in a dose dependent way. Total inhibition is only seen when the samples are incubated in plastic tubes. In the presence of glass there is a persistent activity that can be attributed to a thrombin independent cold activation caused by the contact phase of blood coagulation. The inhibition by hirudin of cold activation in plastic tubes indicates that thrombin is involved especially if contact activation is (neglectibly) low. The mechanism by which thrombin activates factor VII cannot be derived from this experiment, of course. As could be expected, no factor VII activation is observed when the experiments depicted in Figs 7 and 8 are carried out with factor XII deficient plasmas.

In order to track down a possible effect of thrombin upon the contact phase of blood coagulation, we determined the kallikrein formation during incubation of normal pooled plasma at 4°C in glass and plastic tubes in the absence and presence of hirudin (Table I). It is evident from Table I that the presence of hirudin in the incubation mixture does not influence the generation of kallikrein as measured with the chromogenic substrate S2302.

Table I. The effect of hirudin on the generation of prekallikrein activity during incubation of normal plasma at 4°C

Incubate composition	Velocity of S2302 hydrolysis (0.1 AU/min)		
	0 h incubation	4 h incubation	24 h incubation
Buffer (G, P)	—	—	—
Hirudin in buffer (G, P)	—	—	—
Normal plasma + buffer (P)	—	—	0.1
Normal plasma + hirudin (P)	—	—	0.1
Normal plasma + buffer (G)	—	0.2	0.3
Normal plasma + hirudin (G)	—	0.2	0.3

Normal plasma is incubated in the presence or absence of hirudin (final concentration 100 µg/ml); the prekallikrein generation is measured with S2302. G: incubation in glass; P: incubation in plastic.

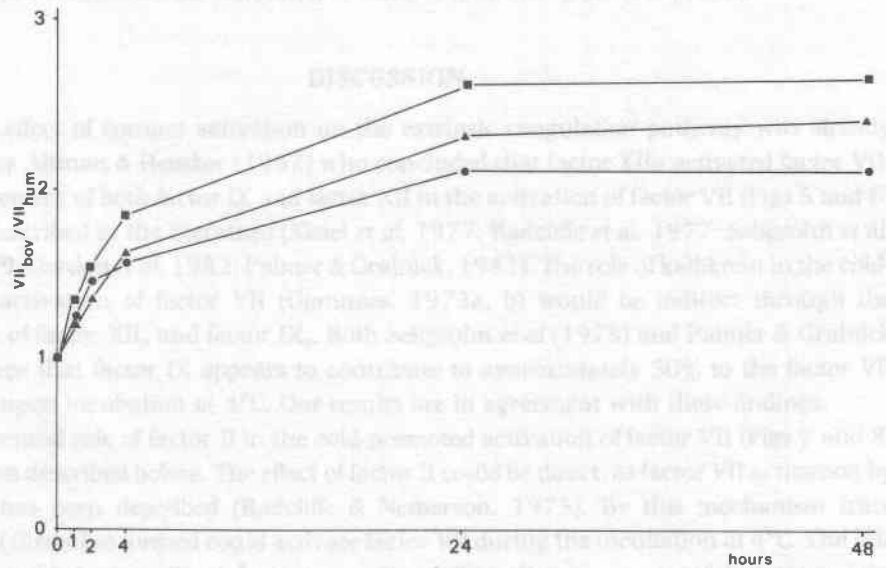


Fig 9. The ratio VII_{bov}/VII_{hum} in normal plasma incubated at $4^{\circ}C$ in glass tubes in the presence of different thrombin concentrations. ●, Normal plasma; ▲, 10^{-3} U thrombin/ml normal plasma; ■, 10^{-2} U thrombin/ml normal plasma.

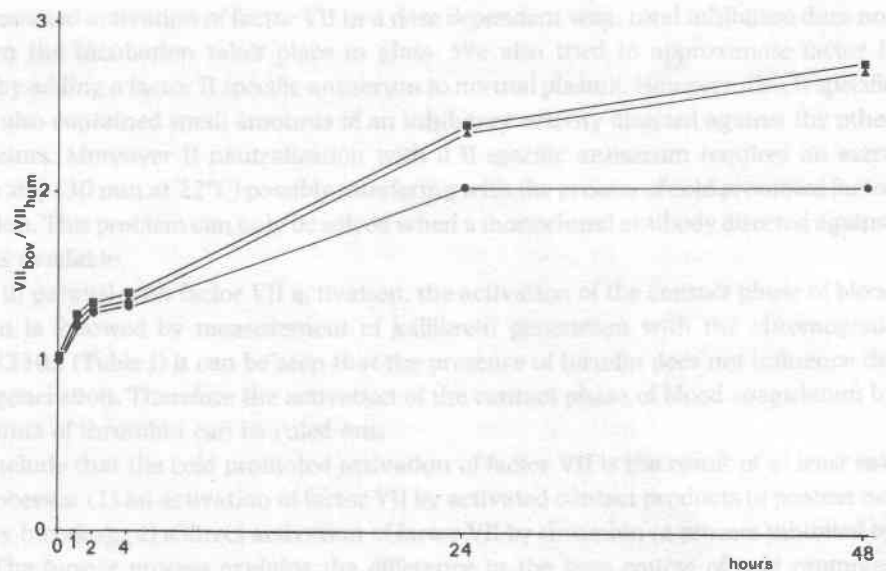


Fig 10. The ratio VII_{bov}/VII_{hum} in normal plasma incubated at $4^{\circ}C$ in plastic tubes in the presence of different thrombin concentrations. ●, Normal plasma; ▲, 10^{-3} U thrombin/ml normal plasma; ■, 10^{-2} U thrombin/ml normal plasma.

From Figs 9 and 10 it is evident that the presence of thrombin in the incubation mixture stimulates the cold promoted activation of factor VII in both glass and plastic.

DISCUSSION

The direct effect of contact activation on the extrinsic coagulation pathway was already described by Altman & Hemker (1967) who concluded that factor XIIa activated factor VII. The involvement of both factor IX and factor XII in the activation of factor VII (Figs 5 and 6) has been described in the literature (Kisiel *et al*, 1977; Radcliffe *et al*, 1977; Seligsohn *et al*, 1978, 1979; Gordon *et al*, 1982; Palmer & Gralnick, 1983). The role of kallikrein in the cold-promoted activation of factor VII (Gjønnaes, 1972a, b) would be indirect through the generation of factor XII_a and factor IX_a. Both Seligsohn *et al* (1978) and Palmer & Gralnick (1983) agree that factor IX appears to contribute to approximately 50% to the factor VII activation upon incubation at 4°C. Our results are in agreement with these findings.

The essential role of factor II in the cold-promoted activation of factor VII (Figs 7 and 8) has not been described before. The effect of factor II could be direct, as factor VII activation by thrombin has been described (Radcliffe & Nemerson, 1975). By this mechanism trace amounts of thrombin formed could activate factor VII during the incubation at 4°C. The role of factor II could also be indirect, for instance via an effect of trace amounts of thrombin on the contact phase of blood coagulation.

In order to study the effect of factor II on the process of cold promoted activation, factor II deficiency was approximated by adding the thrombin inhibitor hirudin to normal plasma. As can be seen in the Figs 7 and 8, the addition of hirudin to the incubation mixture slows down the cold promoted activation of factor VII in a dose dependent way; total inhibition does not occur when the incubation takes place in glass. We also tried to approximate factor II deficiency by adding a factor II specific antiserum to normal plasma. However, this II specific antiserum also contained small amounts of an inhibitory activity directed against the other clotting factors. Moreover II neutralization with a II specific antiserum requires an extra incubation step (30 min at 22°C) possibly interfering with the process of cold promoted factor VII activation. This problem can only be solved when a monoclonal antibody directed against thrombin is available.

When, in parallel with factor VII activation, the activation of the contact phase of blood coagulation is followed by measurement of kallikrein generation with the chromogenic substrate S2302 (Table I) it can be seen that the presence of hirudin does not influence the kallikrein generation. Therefore the activation of the contact phase of blood coagulation by trace amounts of thrombin can be ruled out.

We conclude that the cold promoted activation of factor VII is the result of at least two parallel processes: (1) an activation of factor VII by activated contact products (a process not inhibited by hirudin); (2) a direct activation of factor VII by thrombin (a process inhibited by hirudin). The former process explains the difference in the time course of cold promoted activation of factor VII in glass and plastic tubes; contact activation is more pronounced on glass than on plastic surfaces. The latter process explains the effect of hirudin on the cold promoted activation of factor VII.

When plasmas from normal individuals are incubated at 4°C all samples show cold-promoted activation of factor VII upon incubation in glass; only 25% shows cold promoted factor VII activation upon incubation in plastic (results not shown). These results can be explained by the contribution of the contact phase of blood coagulation. We now wonder if we might speculate that 'cold activators' do occur because of the generation of (trace amounts of) thrombin *in vivo*.

To study the mechanism of cold promoted activation, all congenital factor deficient plasmas were incubated in both glass and plastic. The fact that several congenital factor deficient plasmas show a more or less reduced cold promoted activation of factor VII upon incubation in plastic whereas a normal factor VII activation is reached upon incubation in glass, most probably must be explained by an impaired thrombin generation in the former situation (*in vivo* or *in vitro*).

The involvement of factor II in the cold-promoted activation could also explain the results of Donaldson (1984). This author describes that there is not a clear relationship between Cl-inhibitor deficiency and cold-enhanced plasma coagulation and suggests that events which initiate the process of cold-enhanced clotting activation *in vitro* have already occurred *in vivo*. The equilibria influencing this tendency vary among patients and possibly in any given patient at different times. This and our own data (van Deijk *et al*, 1983b) make us think that it might be useful to investigate whether the cold-promoted factor VII activation *in vitro* can be used as an early indicator of *in vivo* changes in the clotting system as a result of pathological processes. Studies on this subject are in progress.

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Factor VII activation has been described to occur in circulation plasma in defined pathological situations (Ostle *et al.* 1957; Almqvist, 1962; van Dorp *et al.* 1981a, b). We therefore used the test described by Hemker *et al.* (1976) for the *in vitro* assay of activation of factor VII in plasma.

Human factor VII is a single chain protein that can be converted to factor VII_a, a two chain protein. This conversion is associated with an increase in activity as measured in a one stage test. The increase, however, is dependent on the type of thromboplastin used to initiate the reaction. Human tissue-thromboplastin interacts only human factor VII, but hardly with human factor VII while human Streptokinase interacts with both human factor VII and factor VII_a. The increase in activity with conversion of the single chain into the double chain form will therefore be much more pronounced when tested with bovine thromboplastin than with

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